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Transport studies in plasma membrane vesicles isolated from renal cortex

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Transcellular transport in renal tubules involves the movements of solutes between three compartments: luminal, intracellular and peritubular. They are separated by two barriers: the apical (luminal, brush border) and the basolateral (contraluminal, peritubular, serosal) plasma membranes. For vectorial transport of solutes a functional polarity of the cell is required, that is, the transport properties of the apical and the basolateral membrane must be different. Indeed, the two membranes are different in almost every respect: morphology, enzyme content, protein–, lipid–, and carbohydrate–composition, hormone receptors and transport properties. These differences were exploited in the procedures for membrane separation. Different lipid–to–protein ratios and different carbohydrate contents result in different physical properties of the two membranes: density and surface charge, which allow for their separation by density gradient centrifugation, phase partitioning, differential precipitation and electrophoresis. Differences in enzyme activities provide the criteria for identification of the separated membranes.

Isolation of vesicles formed by the brush border and the basolateral membranes made it possible to characterize a number of transport systems under well–defined in vitro conditions. In studies with vesicles it is documented that the transcellular transport of solutes is catalyzed by an array of transport systems located asymetrically in the two membranes. Typically, the transport of a given solute is energized in one membrane only, whereas the other membrane allows for its transport down the chemical or electrochemical potential gradient. The input of energy is necessary not only to create transepithelial or transmembrane concentration gradients, but also to determine the direction of transport.

Two types of energization are usually distinguished: in primary active transport, the chemical energy of ATP is used directly by transport ATPases to drive transmembrane solute transport (such as, Na, K-ATPase, Ca-ATPase, H-ATPase), whereas in secondary active transport, the ion gradients established by the ATPases are utilized to drive the uphill transport of solutes via flux–coupling mechanisms (co- and countertransport). Studies with membrane vesicles isolated from renal and intestinal epithelium were pivotal in establishing the stoichiometry, mechanism and kinetics of several Na-coupled co- and countertransport systems [1–21].

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A brief survey of 13 years of studies with vesicles

Before entering into a discussion of "do's" and "don't's" in vesicle methodology, we would like to mention in the two following paragraphs the transport studies of a variety of substrates which had been made in plasma membrane vesicles isolated from renal proximal tubules. Since the present review deals mainly with the methodological aspects of vesicle studies, no attempt will be made to present a comprehensive picture of different transport pathways as they emerge from the vesicle experiments. It must be noted that for critical evaluation of the evidence for the existence (cross–contaminations?), tubular localization (heterogeniety?) and physiological role of a transport system, not only a careful examination of vesicle data, but also a comparison with the data obtained in the intact epithelium (microperfusion, electrophysiology) is required. Such a detailed examination of each transport system is beyond the scope of this review; for the respective discussions the reader is referred to several topical reviews [12, 16, 17, 20, 161] and to the original papers enumerated below.

In studies with brush border membrane vesicles, a number of different Na-coupled cotransport systems were identified: at least two different systems for hexoses [22–81], several systems for the different groups of amino acids [26, 50, 65, 67–69, 82–125], several systems for organic anions such as bile acids, aromatic acids, metabolites [17, 126–161], separate systems for phosphate [5, 17, 20, 27, 45, 65, 69, 162–209] and sulphate [17, 20, 210–213]. Recently Na-coupled transport of adenosine has been reported [223]. Evidence for Na-independent amino acid transport—mainly for the basic amino acids—was also presented [84, 111, 114, 121, 214, 215]. Peptides are transported via H-coupled transport systems [216–222]. Another group of transporters catalyze the exchange reactions across the brush border membrane; the most prominent example is the Na/H exchange [2, 3, 175, 224–250]. Evidence for exchange systems for inorganic anions [17, 227, 247, 251–253] and organic anions [17, 46, 254–258] was also obtained in studies with brush border vesicles. Experimental support for the existence of primary active H-secretion has been presented although the cellular localization of this mechanism (cross–contamination?) has not yet been established [259, 260, 369, 370]. An electrogenic saturable pathway for Ca-entry has been described [261, 262]. Finally, a

Table 1A. Studies on major transport pathways in isolated brush border membrane vesicles

Mechanism	Physiological function
1) Na-solute cotransport systems	
— hexoses	reabsorption
— L-amino acids (different systems for different groups of amino acids)	reabsorption
— organic acids (different systems for different groups of organic acids, e.g. mono-, ditricarboxylates, bile acids, aromatic acids)	reabsorption
— phosphate	reabsorption
— sulphate	reabsorption
2) Na-H exchange	H secretion, HCO_3-reabsorption
3) H-peptide cotransport	reabsorption

Table 1B. Studies on major transport pathways in isolated basolateral membrane vesicles

Mechanism	Physiological function
1) Na-independent systems:	cellular exit in tubular reabsorption
— hexoses	
— phosphate	cellular uptake for metabolism
— sulphate	
— L-amino acids	
2) Na-solute cotransport systems:	
— L-amino acids	
— gluthathione	
— organic acids (different systems for di/tricarboxylates)	cellular uptake for metabolism
— PAH	cellular uptake in tubular secretion
3) Na-Ca-exchange	maintain of low cellular (Ca); cellular exit in reabsorption
4) Primary active pumps:	
— Na-K-ATPase	maintain of low cellular (Na)
— Ca-ATPase	maintain of low cellular (Ca); cellular exit in reabsorption

few reports deal with transport of other substances, such as organic bases [264–265] and ATP [266].

The transport systems in the basolateral membranes serve at least two different purposes: i) they are required for the influx or efflux of solutes which are transcellularly transported; ii) similar to the transport systems in the plasma membrane of non-polarized cells, basolateral transport mechanisms are required to keep the transmembrane ion gradients, such as Na, K-ATPase and Ca-ATPase, or to allow the entry of metabolites. For D-hexoses a Na-independent pathway was described [44, 68, 267]. Evidence for Na-independent transport of phosphate [183, 268–270], sulphate [268, 271–273] and organic anions [129, 272–276] was obtained in studies with basolateral membranes. For L-amino acids Na-dependent and Na-independent mechanisms were described [68, 87, 104, 116, 277, 281]. A Na-dependent pathway for glutathione was described [277, 278]. Separate Na-dependent transport mechanisms for di/tricarboxylates [139, 282] and for PAH [137, 274, 283–288] have been identified. There is evidence that sodium is transported in the basolateral membranes by two mechanisms, the classical ouabain–sensitive Na, K-ATPase and a separate ouabain–insensitive Na, ATPase [289–293]. Similarly, two mechanisms for Ca-efflux exist: the calmodulin–dependent Ca-ATPase and a Ca/Na exchange [294–303]. Finally, it should be mentioned that one group provided evidence for Na-coupled phosphate flux in basolateral membranes [269, 270].

In Table 1, the major groups of transport systems located in the brush border and the basolateral membranes are listed. This is not a complete list, since only those systems are included which have been extensively documented in both in vivo and vesicle studies.

Although the past 13 years of studies with isolated renal plasma membrane vesicles documented the power of this method and provided a wealth of information concerning the existence, localization, and properties of a number of transport systems (Table 1), numerous limitations, pitfalls and considerable potential for producing artifacts became equally apparent. In the following, we will try to outline briefly some strategies and methods used in the isolation of the brush border and the basolateral membranes and in the analysis of their transport properties. In the present review, we will concentrate on the difficulties and uncertainties which are encountered in vesicle studies, and which are not always recognized in experimental work. We would like to point out that despite of this focus on the "negative aspects" of work with isolated vesicles, we are convinced that experiments with vesicles represent a very powerful tool for the analysis of the membrane transport mechanisms involved in proximal tubular transport of different solutes. Our discussion intends to be complementary to the reviews published before and concentrating mostly on the "positive aspects" [1–21]. It is fair to indicate that in most reports on transport studies with renal cortical vesicles the criteria for the identification of a transport pathway were fulfilled, although a considerable amount of work remains to be done for their complete characterization, and in some cases also for their localization into the brush border–, basolateral– and/or an intracellular membrane.

Methods of membrane isolation

The selection of a method for membrane isolation is usually dictated by the aim of the study. For studies of kinetics, energetics, stoichiometry, biochemical identification/modifications and reconstitution of a transport system, a fast isolation method providing high yield of sealed vesicles containing the intact transport system is of primary importance. Small cross–contaminations with other membranes may be acceptable, as long as the cross–contaminating structures do not contain high activities of the same or a similar transport system. For localization studies, on the other hand, a method which separates effectively *all* the membrane structures which might contain the transport system under study is mandatory; yield and membrane integrity might be of secondary importance. In general, one can distinguish between analytical isolation procedures which usually separate the membranes according to different physical criteria (density distribution, charge distribution, phase partitioning) and which yield small amounts of highly purified membrane fractions, and preparative procedures which allow for fast separation of large amounts of membranes according to one criterium (such as density) whereby purity is sacrificed for the sake of speed and yield.

The precautions to be taken during membrane isolation can be summarized as follows.

Prevention of membrane damage

Membrane constituents undergo a constant turnover. Upon disruption of the cell the syntheses are stopped, but the breakdown of membrane constituents (proteolysis, phospholipid hydrolysis) is not or is even accelerated because of the liberation of degrading enzymes (such as lysosomal). Proteolytic damage may be reduced by using protease inhibitors. Phospholipid hydrolysis can be reduced by removing calcium (addition of EGTA) and adding magnesium to the isolation medium. Oxidative damage can be reduced by free radical scavengers (such as mannitol at high concentrations) and by reducing agents (such as dithiothreitol). However, it can be safely assumed that all isolated membranes are to some extent damaged, the degree of damage depending on the precautions taken and on the duration of the isolation procedure. Therefore, even when working in the cold, speed is an essential condition for successful membrane isolation.

Homogenization

Due to rigid cytoskeletal structure, the brush border region of epithelial cells is relatively resistant to homogenization, allowing for the isolation of large fragments of the apical cell structures which are then disrupted to form smaller membrane vesicles, the brush border vesicles [304–308]. The brush borders withstand even very aggressive homogenization procedures: the membranes isolated from kidney cortex homogenized with Polythron still show structures resembling intact microvilli and are filled with electron-dense cytoskeletal material [309–311]. In general, for isolation of basolateral membranes, more gentle homogenization procedures (such as nitrogen cavitation, Potter, Dounce) are required [313–315]. Homogenization of either the tissue or the isolated basolateral membrane vesicles with Polythron results in an inactivation of both Na, K-ATPase and Ca-ATPase (P. Gmaj, H. Murer, unpublished observation). The possibility of forming particles of different size and density, with more or less of cytoskeletal material and more or less of inactivated marker enzymes implies that the homogenization procedures must be strictly standardized. It must be checked if the marker enzymes are not inactivated—otherwise even large cross-contaminations may go undetected.

Choice of membrane markers: consequences for the localization of a transport system

When the activities of membrane-bound enzymes are used to follow the isolation, special attention must be paid to a balance sheet (recoveries) of total enzyme activities in the different fractions: inactivation of an enzyme should be prevented and cryptic activity (due to inaccessibility of substrate binding sites located inside the vesicles) should be detected. Na, K-ATPase, Ca-ATPase and hormone-stimulated adenylate cyclase are usually used as markers for basolateral membranes; alkaline phosphatase, γ-glutamyltranspeptidase, aminopeptidase M and different disaccharidases (such as maltase, trehalase) are used as markers for the brush border membranes [9–16, 24, 25, 65, 109, 260, 267, 303–322]. However, one must be aware that marker

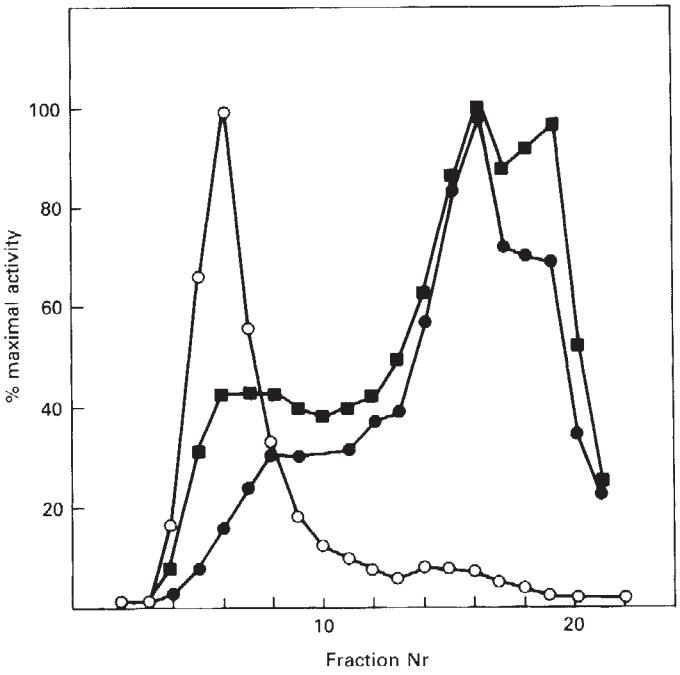

Fig. 1. Separation of rat kidney cortex microsomes on a Percoll gradient. Microsomes from outer kidney cortex were prepared as described [310]. Centrifugation in 10.4% Percoll for 35 min at 48,000 × g. Symbols are: (■-■) γ-GT, gamma-glutamyltranspeptidase; (●-●) LAP, leucine-aminopeptidase; (○-○) Na, K-ATPase.

enzymes might not be localized in one membrane exclusively. Membrane traffic involved in the turnover of membrane constituents and in endo- or exocytosis makes an exclusive localization rather unlikely. Multiple localization does not preclude the use of an enzyme as a marker, as long as the isolation procedure separates the different membranes which contain the same enzyme activities [15]. The yields, recoveries and enrichment factors can be easily misinterpreted when considerable amounts of marker enzyme activities are present in membranes other than the membrane under study.

Multiple localization of marker enzymes also complicate the assessment of cross-contaminations. Some of these problems are illustrated in Figure 1 which shows the separation of kidney cortex microsomes on a self-orienting Percoll gradient. The marker for basolateral plasma membranes Na, K-ATPase is clearly separated from brush border markers, aminopeptidase M and γ-glutamyltranspeptidase. However, all basolateral plasma membrane fractions contain some activity of brush border markers and vice versa. This could indicate that i) the membranes are cross-contaminated; ii) the basolateral membranes contain some γ-glutamyltranspeptidase activity whereas the brush border membranes contain some Na, K-ATPase activity; iii) another vesicle population is still present in the mixture which contains both enzymes and is not separated on the gradient (vesicles involved in the membrane traffic, plasma membrane vesicles from a different nephron segment, or vesicles from the same segment but from a different nephron population). If the membranes are cross-contaminated, then the degree of cross-contamination of basolateral membranes

Table 2. Separation of membranes containing Na-Pi cotransport system from the bulk of basolateral membranes[a]

	Basolateral membranes	Electrophoretic fractions		
		1	2	3
Na-Pi cotransport (% stimulation by Na^+ of initial uptake)	106 ± 23	26 ± 23	87 ± 3	162 ± 17
Na, K-ATPase (mU/mg protein)	1247 ± 380	1793 ± 28	1233 ± 28	761 ± 86
Leucine–aminopeptidase (mU/mg protein)	125 ± 11	39 ± 3	61 ± 6	251 ± 51

[a] Basolateral plasma membranes were isolated from rat kidney cortex by Percoll gradient centrifugation, and separated further into three fractions by free-flow electrophoresis. Values are means ± SE, $N = 4$ (from Hagenbuch and Murer, unpublished).

will be much higher when assessed on the basis of γ-glutamyl-transpeptidase rather than of aminopeptidase M activity.

It is obvious that the above mentioned problems cannot be resolved by measuring marker enzyme activities in a single separation procedure. A second separation, based on different physical principles is necessary (free flow electrophoresis after differential and/or density gradient centrifugation or vice versa, or differential and/or gradient centrifugation in combination with phase partitioning [15, 240, 322]. The consequences of the problem with cross-contaminations for the localization of a transport system are illustrated in the experiment presented in Table 2. In basolateral membranes isolated from rat kidney cortex by a Percoll-gradient centrifugation and enriched about twentyfold in Na-K-ATPase activity, a Na-stimulated phosphate transport can be demonstrated (Hagenbuch and Murer, unpublished observations). When, however, the membranes are separated further by free flow electrophoresis, no significant Na-dependent phosphate transport is found in fractions with highest activities of Na, K-ATPase and ATP-dependent Ca-transport, but it is found in fractions enriched in aminopeptidase M and in sodium-dependent D-glucose transport (Table 2; Hagenbuch and Murer, unpublished observations). This analytical experiment demonstrates that, at least in the rat, there is no Na-dependent phosphate transport in the basolateral membranes, although such transport mechanism could be inferred from experiments with membranes isolated by a preparative procedure. Therefore, the extent of purification (enrichment factor) is in studies on the localization of a transport mechanism (brush border vesicles vs. basolateral) only of secondary importance; it is more important to follow in at least two subsequent separation procedures the transport activity and to compare it with known properties of a given membrane. A high enrichment factor does not a priori exclude cross-contamination.

Cellular origin of the isolated membranes; tissue heterogeneity

The kidney is a highly heterogenous tissue, and so are the membranes isolated thereof. The heterogeneity can be reduced by careful anatomical separation of tissue zones before proceeding to membrane isolation. The outermost cortex contains mostly the proximal convoluted tubules of superficial nephrons; juxtamedullary cortex contains mainly the proximal tubules of deep nephrons and the pars recta of the superficial nephrons; the outer medulla consists mostly of pars recta of both nephron populations.

Anatomical pre-separation of kidney tissue before membrane isolation allows the detection of heterogeneity of transport systems in different nephron segments: both qualitative and quantitative differences in Na-coupled transport systems for glucose [75–78], phosphate [168, 323], lactate [140], L-amino acids [78, 104, 114] and in Na/H exchange [324] were observed in brush border membranes isolated from superficial and juxtamedullary kidney cortex. These experiments provided the most compelling evidence for heterogeneity of transport systems for the same solute within the proximal tubule.

In contrast to the brush border membrane, isolation procedures based on selective isolation criteria, such as the well-developed brush border structures (caps) and/or the specific external membrane surface composition (divalent-cation precipitation), no selective method is available at present for isolation of basolateral membranes from different cells. Therefore it must be assumed that basolateral membranes represent a mixture of plasma membranes from all the cells present in the starting tissue. The origin of the bulk of basolateral membranes should be established by measuring hormone stimulation of adenylate cyclase. In membranes isolated in our laboratory from outer rat kidney cortex the presence of PTH activation and the absence of vasopressin activation of adenylate cyclase suggests their proximal tubular origin [314, 325].

Specific techniques for isolation of brush border and basolateral membranes

The earlier methods to prepare the brush border membranes were based on a location of large membrane fragments called brush border caps [304–308]. At present, the most frequently used methods for brush border isolation are based on differential precipitation of subcellular organelles with calcium or magnesium [246, 309, 311, 326–329]. Due to the high negative surface charge densities in brush border membranes, the addition of divalent cations does not lead to their precipitation with other cellular membranes [326]. Compared to calcium-precipitation, magnesium precipitation in calcium-free solutions yields brush border membranes with considerably reduced leak permeabilities, which is probably related to the presence of calcium-dependent hydrolytic enzymes [246, 309]. In brush border membranes isolated by calcium precipitation, the Na-gradient-dependent proton movements were in part produced by electrodiffusional coupling between the fluxes of the two ions; in membranes isolated by magnesium precipitation, the electrodiffusional movements of Na and H were minimal, and the fluxes of the two ions were mostly coupled by electroneutral Na/H exchange [246].

Brush border membrane vesicles are oriented right-side out [310]. It has not yet been possible to obtain inside-out oriented brush border membrane vesicles, even when very harsh physical methods were applied, such as osmotic shock, ultrasound or French press (H. Murer, unpublished observation). The resistance of brush border membranes to inversion is probably related to their architecture: the presence of cytoskeleton inside the vesicles, dense packing of enzymes, and high density of

negative charges on the outer membrane surface. In intestinal brush border membranes, it is possible to remove large amounts of non-membrane proteins without destroying transport function by extraction with high concentrations of KSCN [81, 124, 330]. Application of this method to renal membranes resulted in an inhibition of transport which, however, could be restored by partial reconstitution and repair of the bilayer (P. Gmaj and H. Murer, unpublished observation).

Basolateral plasma membranes are usually isolated from microsomes obtained by differential centrifugation. Density gradient centrifugation in different media and free flow electrophoresis were used for further membrane separation [15, 16, 44, 109, 137, 233, 265, 275, 277, 280, 281, 290, 298, 303, 313–316, 321]. Centrifugation in self-orienting Percoll gradients provides an especially fast and easy procedure [280, 298, 314, 321]. In contrast to brush border membranes, the basolateral membranes are in general not uniformly oriented and their sealing is incomplete. The vesiculation and the orientation of the basolateral vesicles were studied by electron microscopy, by the measurement of the detergent activation and the increase in ouabain-sensitivity of Na, K-ATPase, by measuring the cryptic ouabain-binding sites and by comparing the ATP-dependent Ca transport with Ca-dependent ATP hydrolysis [289, 291, 299, 303, 310, 315, 331]. The heterogeneity of basolateral membranes with respect to cellular origin, as well as to orientation and to sealing, must be considered in the interpretation of transport studies. Several attempts to separate the inside-out, right-side out and open vesicles, such as on lectin-columns, have been unsuccessful (H. Murer, unpublished observation). In our laboratory the only method which improved the degree of vesiculation and tightness of the basolateral membranes was a partial solubilization and reconstitution [332].

Biochemical characteristics of the brush border and the basolateral membranes

Enzyme activities

A lot of information is available on the enzyme content of the brush border and basolateral membranes. Here we would like to mention only those which are of use for membrane identification and those for which a transport or a regultory function was postulated.

Disaccharidases (maltase, trehalase) and peptidases (γ-glutamyltranspeptidase, aminopeptidase M) and alkaline phosphatase are commonly used to identify the brush border membranes; Na, K-ATPase, Ca-ATPase and hormone sensitive adenylate cyclase are usually used to identify the basolateral membranes [9, 11–13, 15, 16, 18].

The brush border vesicles contain enzymes which may play a role in regulating transmembrane transport. cAMP-dependent and Ca phospholipid-dependent protein kinase activities were measured [12, 165, 178–181, 333, 336]. Also, evidence for protein ribosylation [176, 179, 337, 338] and lipid methylation was presented [339, 340]. The involvement of these regulatory enzymes in altering transmembrane flux of phosphate and calcium was postulated, and at least the data concerning phosphate transport are under dispute [165, 176, 178, 179].

A role of alkaline phosphatase in the transport of phosphate was discussed but it seems highly unlikely today. There is also no evidence for a participation of γ-glutamyltranspeptidase in transmembrane amino acid transport in renal brush border membranes. In both instances, experiments with vesicles provided useful information [65, 96, 99, 187, 194, 201, 209]. The role of Na, K-ATPase and Ca-ATPase in transport is evident. The function of the different hydrolytic enzymes in the brush border membrane might be related to a degradation of oligomers to form the units to be transported.

Table 3. Lipid composition of the brush border and basolateral membranes

	Brush border	Basolateral
Cholesterol, *nmole/mg protein*	552 ± 9.5	397 ± 15.9
Phospholipid, *nmole/mg protein*	546 ± 13.9	712 ± 37.0
Individual phospholipids, %		
Lysophosphatidylcholine	0.68 ± 0.05	0.27 ± 0.06
Sphingomyelin	34.05 ± 1.1	13.4 ± 0.7
Phosphatidylcholine	16.2 ± 0.6	38.4 ± 1.6
% plasmalogen[a]	0.4 ± 0.05	0.5 ± 0.1
Phosphatidylethanolamine	30.0 ± 0.9	32.3 ± 0.9
% plasmalogen[a]	22.8 ± 3.2	11.5 ± 2.1
Phosphatidylinositol	1.75 ± 0.12	4.31 ± 0.49

[a] Plasmalogens are given as percent of parent compounds.

Lipid composition

The lipid composition and the relative phospholipid turnover rates in the brush border and the basolateral plasma membranes were recently studied by Molitoris and Simon [341]; their data are shown in Table 3. Characteristic differences involve a much higher content of cholesterol, sphingomyelin, and plasmalogen in the brush border as compared to the basolateral membranes. The relative rates of phospholipid turnover are much higher in the basolateral membranes. Changes in phospholipids have been associated with altered brush border membrane calcium transport [262, 339, 340, 342]. The lipid composition influences membrane fluidity and thus affects transport [340, 343–348].

The protein and carbohydrate composition of renal brush border and basolateral membranes has not been studied in detail as yet. To our knowledge, all studies of protein composition published so far were done in membrane preparations which had not been purified from non-membrane constituents (cytoskeleton, aggregates, etc.) and therefore do not reflect the composition of the membranes themselves. For example, one of the most prominent protein bands found in isolated brush border membrane vesicles is actin, a component of intravesicular cytoskeleton [18, 65, 349].

Techniques of transport measurements

Tracer flux measurements followed by rapid filtration of the vesicles is the method used most frequently to study transport in isolated plasma membrane vesicles [16, 133]. By using a semi-automatic mixing/diluting apparatus it is possible to measure the uptakes at times shorter than 1 second [122]. The separation of the vesicles from the incubation medium must be done without significant loss of intravesicular substrate and without significant retention of extravesicular substrate on the filter. This is done by quenching the transport reaction with large volumes of ice-cold, iso- or hypertonic "stop solution,"

which may contain an inhibitor of the transport system under study [133]. However, the loss of substrate during dilution and filtration must be checked in preliminary experiments.

Substrates may bind to the vesicles in addition to being transported. External binding is usually very fast and is indicated by uptake curves which do not extrapolate to zero uptake at time zero [82, 122]. Internal binding can only be evaluated at equilibrium, in the following ways: (i) the distribution space of the substance under study can be compared to that of a substance which is not bound; and (ii) the distribution space of the substance is measured at different extravesicular osmolarities established with non-penetrant solute, such as sucrose. It is assumed that in the absence of binding the distribution space extrapolates to zero for infinite osmolarity [44, 122]. External binding can be reduced, such as by chasing the isotope by an excess of non-radioactive substance present in the "stop solution", or, as in the case of calcium, by including a complexing agent (EGTA) in the stop solution [297, 299, 350]. In the case of lipophylic compounds, however, binding and diffusion can hardly be distinguished from specific transport in tracer flux experiments. Rapid filtration can be replaced by ion exchange chromatography, gel filtration [351] and by centrifugation [22, 84]. The precautions to be taken with these techniques are exactly the same as with the rapid quenching/filtration method.

Some transport processes can be studied by optical methods. Electrogenic transport systems can be analyzed by means of charged hydrophobic dyes (such as cationic cyanine derivatives) and either fluorometry or dual wavelength spectrophotometry [21, 29, 67, 144, 150, 352, 353]. Despite methodological difficulties (calibration, effects of pH and ionic strength on fluorescence, etc.) these methods proved most useful for direct demonstration of electrogenicity of transport systems in isolated membrane vesicles. Transport processes involving H^+ movements across the membrane, H^+ conductance and Na^+/H^+ exchange, can be studied by means of the pH-sensitive fluorescent dye acridine orange [227, 232, 233, 238, 243–247, 250, 354, 355]. Osmotic flow of solutes can be followed by measuring light scattering, which detects changes in vesicular volume [356]. Compared to tracer methods, the optical methods have several advantages: (i) high time resolution which allows for the measurement of fast transport systems; (ii) no disequilibrium is created when the vesicles are separated from the incubation medium; (iii) the results can be evaluated immediately which increases experimental flexibility; and (iv) low running costs. The disadvantages include difficulties in controls for unspecific effects and in quantitative calibration, requirements of large amounts of vesicles, and a limited range of transport systems which can be studied by these methods.

Criteria used to establish the existence of a transport system

The existence of a specific transport system is inferred from the properties of a transport process.

Saturability. Transport via a specific transport system is expected to be saturable with respect to substrate (such as glucose), cosubstrate (such as Na^+), activators (such as ATP) and modulators (such as calmodulin). Measured under initial rate conditions, simple diffusion is not saturable. Hence, nonlinearity in saturation experiments suggests mediated transport.

Specificity. The specificity of a transport system is usually evaluated in two ways: (i) by measuring the transport of structurally related substrates, such as D/L-glucose, L/D-amino acids and their analogs; and (ii) by measuring the interference of non-radioactive analogs with the transport of the radioactive substrate. The inhibition of tracer uptake by non-radioactive substrate or its analog present on the same membrane side (cis-inhibition) and the stimulation of tracer uptake by non-radioactive substrate or its analog present on the opposite membrane side (trans-stimulation) provide information about tracer coupling, that is, about substrates which are transported simultaneously by the same transport system [145, 258, 268, 272]. It must be noted, however, that apparent cis-inhibition and trans-stimulation may also result from indirect effects, such as competition for driving forces, cosubstrates or activators [27, 60, 69].

Temperature dependence. The activation energy and hence the temperature dependence of catalyzed transport is much higher than that of simple diffusion. In addition, the activity of a membrane–bound transport system is usually a non-linear function of temperature: two slopes are observed on Arrhenius plots of ln v vs. 1/T. This is due to a change in activation energy of a transport system upon phase transition of membrane lipids from solid to liquid state [33, 167, 343–345].

Specific inhibitors. Some transport systems can be inhibited specifically, such as Na, K-ATPase with ouabain [289], Na-glucose cotransport with phlorizin [22–25, 44], Na-independent glucose transport with phloretin or cytochalasin B [44, 68], and ATP-dependent Ca transport with vanadate [299]. For most transport systems, however, no specific inhibitors are available. With certain precautions, unspecific inhibitors (such as mercurials) can also be used to block mediated transport and thus to distinguish it from simple diffusion [129].

Flux coupling. A number of transport systems catalyze simultaneous translocation of more than one substrate in either co- or countertransport mode. The criteria to establish a cotransport system were recently discussed in detail by Turner [19] and by Aronson [1, 4]. With some modifications the same criteria can be applied to countertransport systems. In Na-coupled transport, a Na-gradient should drive the transport of substrate, substrate gradient should drive the transport of Na, and Na should accelerate substrate flux even in the absence of a gradient. If the transport is electrogenic, simultaneous translocation of Na, substrate and electric charge across the membrane must be demonstrated, for example, by measuring relative changes in the membrane potential by potential sensitive dyes, by using ionophores to clamp the membrane potential (valinomycin + K^+) or the Na-gradient (gramicidin D), and by measuring the effects of alterations in membrane potential on the simultaneous movements of Na and substrate. One of the aims of such experiments is to rule out the possibilities of indirect coupling of substrate– and cosubstrate–flux via membrane potential or via pH gradients. The transmembrane pH can be clamped by ionophores (such as nigericin; FCCP and valinomycin) in combination with buffers of high capacities.

Advantages and disadvantges of studies with vesicles

We will mention briefly some of the advantages and disadvantages of studies with isolated membrane vesicles. A more extensive discussion of this subject was presented in a recent review [18].

Separation of membranes from other cell constituents

For the analysis of kinetics and energetics of a transport system it is necessary that the concentrations of substrate and cosubstrate on both sides of the membrane are known—at least at zero time [6–8, 19, 357, 361]. This condition cannot be achieved in intact cells, since practically all substrates are either metabolized, or sequestered, or transported by different transport systems located in different membranes. The absence of metabolism in the isolated membranes vesicles [316], however, must not be taken for granted. Oxidation reactions (such as cysteine), pH-dependent reactions (such as conversion of monovalent to divalent phosphate), a number of hydrolytic reactions (such as hydrolysis of NAD [362, 363], reactions of added substrates with membrane proteins [82, 337, 338], and phospholipids and intravesicular production of a substrate (such as phosphate from phospholipids) can still take place. Thus, identification of the transported substrate in the vesicles, as by chromatography, is often a necessary preliminary experiment.

Separation of the plasma membrane from the rest of the cell distinguishes the regulatory mechanisms which require an intact cellular machinery from those which are inherent in the transport systems under study. Allosteric regulation of Na/H exchange [224], Na-SO_4 cotransport [210, 212], and Na-PO_4, cotransport [162], as well as the involvement of K in Na-dependent transport of acidic amino acids [83, 112, 117–119, 280] were first detected in isolated membrane vesicles. On the other hand, the involvement of membrane bound protein kinase reactions in the regulation of phosphate transport turned out to be difficult to prove because of the necessity to open the closed right–side out oriented brush border membrane, the rapid hydrolysis of the added nucleotides and their interference with transport measurements [165, 176, 178, 179, 193, 362, 363]. The regulatory mechanisms which involve protein synthesis and insertion of new transport systems (adaptive changes) into the membranes cannot be studied in isolated membrane vesicles, although the final effects of these regulations can be detected as a decreased or increased activity of the transport system in the isolated membrane. The analysis of intact cells and isolated membrane allowed to establish that both types of mechanisms, allosteric and adaptive, are involved in the regulation of Na-P_i transport [364].

Localization of transport systems

Separation of brush border and basolateral membranes made the studies of functional polarity of epithelial cells possible and allowed for the separate measurements of the transport system. However, as it has been mentioned before, tissue heterogeneity and unavoidable cross–contaminations require a most careful interpretation of studies with vesicles on the localization of a transport system. For example, the eventual participation of brush border membranes in the measured transport process in a basolateral membrane preparation is usually evaluated by measuring some transport processes characteristic for the brush border membrane, such as Na-dependent glucose uptake [280]. Such controls, however, have only a relative value, since the brush border membranes in the kidney are not homogenous [76, 78, 105, 140, 323, 324] and the properties of brush border copurified with the basolateral membranes might not necessarily be the same as the properties of the bulk brush border fraction. Furthermore, unequal sensitivity in the control and test–system due to different transport rates in the contaminating membrane can lead to a wrong conclusion, that is, an apparent absence of a cross–contamination.

Localization of regulatory events

Altered transepithelial transport can be the consequence of alteration in several mechanisms such as luminal entry, basolateral exit, cellular metabolism/driving force. Studies on vesicles isolated from animals with altered in vivo transport turned out to be most useful. It was shown that most situations of altered phosphate and proton transport are paralleled by altered brush border membrane transport [172, 175, 184, 229, 248, 364, 365]. However, it can only be concluded from such studies that the number of operating transport systems is different when the experiments are performed under initial rates and under saturating conditions (V_{max}), or better, when the transport is analyzed in the entire concentration range and the kinetic constants (apparent K_m and V_{max}) are estimated. Otherwise, it is not possible to distinguish between changes in transport kinetics and changes in the number of operating transport systems.

Interaction between transport systems

Several systems transporting the same substrate may coexist in one vesicle. Because the driving force may deviate fast from the initial conditions, interactions between transport systems may be observed in vesicles which are not present in a viable cell which is able in most of the situations to maintain the driving forces constant via primary active pumps. Such competition for driving force between different Na-solute cotransport systems has been shown in experiments with vesicles [27, 60, 69]. Furthermore, due to the presence of a very active Na/H exchanger in the brush border membranes, a pH-gradient is established whenever the membranes are exposed to Na-gradient unless special precautions are taken (pH-clamp). This may create problems in the measurements of transport reactions which are particularly pH-sensitive. On the other hand, coexisting and functionally coupled transport systems can be dissected in isolated membranes, which is not always possible in the intact cells. For example, in intestinal brush border membranes, the coexistence of Na/H exchange and Cl/OH exchange was documented which provided the explanation of "coupled" electroneutral NaCl cotransport [17].

Leak permeabilities

Passive permeabilities are higher in the isolated membranes than in the intact cells, due to inevitable membrane damage during isolation. This is especially true for ionic conductances, which may be induced by lysophospholipids, free fatty acids, etc. Therefore, the estimation of the relative importance of coupled v. conductive transport is often difficult [246]. Useful indications are provided in those cases by a comparison with electrophysiological data obtained in the intact tissue.

Starting material for isolation and identification of transport systems

Transport systems can be studied in isolated membranes in a partially purified form. Further purification can be obtained by stripping the membranes of nonmembrane constituents [81, 124, 330]. Recent studies have shown that the biochemical identification of transport systems is possible in isolated membranes, such as by using group–specific reagents, by inhibitor binding measurements, or by monoclonal antibodies; partial isolation and reconstitution of some transport systems have already been reported [31–34, 36, 45, 48, 49, 52–58, 61–63, 81, 103, 131, 190, 191, 205, 274, 283, 366–368]. There is no doubt that the investigations of the isolated transport systems will contribute greatly to the understanding of transport mechanisms operating in renal tubular cells.

Conclusions

The isolation of brush border and basolateral membranes from the proximal tubular epithelial cells and the characterization of their transport properties added a new dimension to our understanding of transepithelial solute transport in proximal tubules. The individual membrane transport mechanisms could be studied in each membrane separately and under precisely–known experimental conditions. Mostly on the basis of experiments with isolated membrane vesicles we are able to envisage transcellular transport of a number of substances as a two step process catalyzed by a specific array of transport systems located asymetrically in the brush border and in the basolateral membranes. In most cases, the description of the transport systems at the membrane levels explains satisfactorily the behaviour of transepithelial transport, although sometimes not in full detail.

Some characteristics of the transport mechanisms, such as stoichiometry in flux coupling, substrate specificity, kinetic properties can be studied in great detail in membrane vesicles. Isolated membrane vesicles offer also the possibility to identify and to isolate the molecules involved in the transport. As shown for different transport systems, such as Na/H-exchange and Na-phosphate cotransport, regulatory events in transepithelial transport are in part retained at the isolated membrane level, which offers the chance in a combination with experiments involving intact tissue to understand the cellular mechanisms in transport regulation.

The accumulation of experimental experience over the past 13 years of studies with vesicles inevitably brought into focus also experimental limitations and pitfalls of this technique. In view of the complexity of the starting material, one major concern is related to cross–contaminations either with respect to tissue heterogeneity or to contamination with different cellular membranes. It must be admitted that for some of the intented conclusions we require a more sophisticated methodology, such as in the localization studies prefractionation of the heterogenous starting material and analytical membrane fractionation procedures. For experiments with basolateral membranes a more uniform membrane preparation would be highly desirable.

The reservations which we have put forward in the preceding paragraphs do not intend to invalidate the enormous contribution of this experimental approach to an understanding of proximal tubular transport. After 13 years of personal experience with this technique and in view of the numerous studies published in this time period, we have assumed that clear realization of both the potential and the limitations are useful for the evaluation and for the planning of studies with isolated vesicles.

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